Aquilegia as a Model System for the Evolution and Ecology of Petals

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Abstract

The ranunculid genus *Aquilegia* holds extraordinary promise as a model system for investigating a wide range of questions relating to the evolution and ecology of petals. New genetic and genomic resources, including an extensive EST database, BAC libraries and physical maps, as well as viral induced gene silencing are facilitating this research on multiple fronts. At the developmental genetic level, *Aquilegia* has been important for elucidating the developmental program for specifying petals and petaloid characteristics. Data suggest that duplication events among the petal and stamen identity genes have resulted in sub- and neofunctionalization. This expansion of gene function does not include the petaloidy of *Aquilegia* sepals, however, which does not depend on the same loci that control identity of the second whorl petals. Of special interest is the elaboration of the petal into a nectar spur, a major innovation for the genus. Intra and inter-specific variation in the shape and colour of petals, especially the spurs, has been shown to be adaptative for different pollinators. Thus, understanding the genetic basis of these traits will help us connect the ecological interactions driving speciation with the genetic changes responsible for remodeling morphology. Progress in this area has focused on the multiple, parallel transitions in flower colour and nectar spur length across the genus. For flower colour, upstream transcription factors appear to be primarily targets of natural selection. Thus research in *Aquilegia* spans the initial evolution of petals and petaloidy to the diversification of petal morphology to the ecological basis of petal form, thereby providing a comprehensive picture of the evolutionary biology of this critical angiosperm feature.

Keywords: Aquilegia, Ranunculaceae, petal evolution, adaptation, speciation, nectar spur

1. Introduction

The evolution of petaloid organs in the reproductive axis is a notable innovation of the angiosperms that has clearly played a major role in their diversification, especially in the context of attracting pollinators (Endress 1994). What makes petaloid features so intriguing is that they appear to be a true innovation with no obvious precursor in extant gymnosperms. Then, once petals evolved, more subtle changes to their shape and colour have been major factors allowing species to attract and become specialized on different pollinators. From a genetic standpoint, there are many aspects of petaloid organs that we would like to understand in terms of both macroevolutionary and microevolutionary processes. How many times have petaloid organs evolved? Is their developmental genetic basis the same when petaloid features are produced in different positions? How is the elaboration of petals controlled and how are these genetic pathways altered in the context of pollinator shifts? Do independent shifts to similar pollinators involve similar genetic changes to petals? These types of questions cover a multitude of different genetic pathways and can only be answered through analysis of a similarly diverse set of genetic models. In this review, we discuss the new model system *Aquilegia* and how it is contributing to our understanding of the evolution of petaloid organs.

Flowers typically have two types of organs – reproductive and sterile. While the reproductive organs are divided into stamens (androecium) and carpels (gynoecium), the sterile organs are collectively termed the perianth. The phenomenon of petaloid organs is challenging because it can occur in any of these organs, as well as in extra-floral leaf-like organs termed bracts. If we restrict our consideration to the perianth, there are further distinctions. In some taxa, such as *Magnolia* or *Tulipa*, perianth parts are relatively similar in appearance (whether petaloid or not),

in which case they are termed tepals. More commonly, the perianth is bipartite with two or more distinct organ types (in contrast to the unipartite condition of tepals). In these cases, the outer organs, called sepals, are often adapted for protective functions while the inner organs, called petals, are more specialized for a role in pollinator attraction. Thus, classically, petals have a fixed position on the floral axis – in the second whorl between the sepals and androecium. However, as mentioned above, petaloid features can occur in either of the perianth whorls and in the case of the family Ranunculaceae, of which *Aquilegia* is a member, often occur in both. How then should we define petaloid features? While there is no clear set of criteria (Endress 1994), petaloidy can be defined as being conspicuous and brightly coloured or patterned as opposed to green or leaf-like. It is important to note that in some taxa, the true petals themselves are quite small, meaning that not all petals are dramatically petaloid.

Eames (1961) wrote that "Theories of the nature and development of the perianth are closely bound up with theories of the origin of the flower", by which he was referring to two ideas about where perianth organs came from. According to the first idea, originally espoused by Goethe (1790), the perianth parts, and the petals in particular, are modified stamens termed andropetals (Takhtajan 1991). The alternative hypothesis is that perianth organs were derived from pre-existing sterile bracts that were associated with the reproductive axis, giving rise to bracteopetals (Takhtajan 1991). These two hypotheses may not be mutually exclusive. Some taxa may have perianth organs derived from both bracts and stamens while others may have only bracteopetals or andropetals. The criteria used to distinguish between andro- and bracteopetals are largely developmental and morphological, relating to vasculature, developmental kinetics, phyllotaxy, and morphological similarities (Eames 1961; Smith 1928; Takhtajan 1991). However, beyond

the details, the fundamental point is that the perianth in general and petals in particular have evolved many times independently within the angiosperms, with different precursor organs giving rise to sterile, attractive structures in different lineages (Bierhorst 1971; Eames 1961; Takhtajan 1991). Reconstruction of perianth evolution in the context of modern molecular phylogenies confirms that the bipartite perianth has evolved many different times independently (Hileman & Irish 2009; Zanis et al. 2003), as have novel types of petaloid features in various positions both inside and outside the flower (Jaramillo & Kramer 2007; Walker-Larsen & Harder 2000).

Our understanding of the developmental genetic basis of floral organ development has grown exponentially over the last twenty years, with considerable impact on our understanding and interpretation of floral evolution. Especially notable is the elucidation of the ABC model for floral organ identity (Coen & Meyerowitz 1991). This genetic model describes three classes of gene activity, expressed in overlapping domains of the floral meristem, thereby creating a combinatorial code. Each whorl of the flower has a distinct set of gene activities: A alone encodes sepals; A+B, petals; B+C, stamens, and C alone, carpels (Fig. 1). The model has been modified over time by the addition of two other gene activities (D, for ovules, and E, as a broad facilitator of ABC gene function) and substantial reconsideration of the nature of A function (Davies et al. 2006; Litt 2007). Most of the genes associated with the ABC model are members of the large type II class of MADS box containing transcription factors, which has facilitated their identification across a broad range of seed plants (Becker & Theissen 2003). Overall, it appears that while A function is not conserved, the functions of the B and C class gene homologs

are largely the same throughout the angiosperms, particularly in terms of stamen and carpel identity (Kramer 2006; Kramer et al. 2004; Zahn et al. 2005b).

This leads to the question of petal identity – is there a deeply conserved petal identity program or, in keeping with the idea of multiple independent derivations, are there many genetically different ways to produce a petal? Answering this question has proved difficult, for a number of reasons. First, the B class genes, represented in *Arabidopsis* by the MADS box genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) (Goto & Meyerowitz 1994; Jack et al. 1992), have complex evolutionary histories (Kramer et al. 1998; Kramer & Irish 2000; Stellari et al. 2004) and have experienced gene duplication at every phylogenetic level. Most notably, the *AP3* lineage was duplicated at the base of the core eudicots to give rise to two distinct paralogs termed eu*AP3* and *TM6* (Kramer et al. 1998). This is of interest because the eu*AP3* lineage experienced a distinct sequence divergence at its C terminal end as the result of a frameshift mutation, which converted the otherwise deeply conserved paleoAP3 motif into the core eudicot-specific euAP3 motif (Kramer et al. 2006; Vandenbussche et al. 2003). The biochemical implications of this event are still a matter of debate (Lamb & Irish 2003; Piwarzyk et al. 2007), but the fact remains that the defining B class genes, *AP3* from *Arabidopsis* and its ortholog *DEFICIENS* (*DEF*) from *Antirrhinum*, are members of a core eudicot-specific lineage and therefore do not have simple orthologs outside this clade (Kramer et al. 1998; Kramer & Zimmer 2006). Another complicating factor for understanding the conservation of the petal identity program is the potential for convergence at the genetic level. This phenomenon has frequently been observed among both plants and animals and can result in non-homologous structures expressing homologous genes (Abouheif et al. 1997; Jaramillo & Kramer 2007). Convergence is particularly likely in the case

of andropetals where independent sterilization of stamens could have repeatedly involved the recruitment of B gene homologs to control andropetal identity (Irish 2009). Even for bracteopetals, the transformation of bracts into petaloid organs might have occurred via outward expansion of the B gene domain from the stamens (Baum & Whitlock 1999; Irish 2009). How then should we interpret the finding that B gene homologs are almost always expressed in true petals or petaloid tepals (reviewed in Kim et al. 2004; Kramer & Jaramillo 2005; Zahn et al. 2005b)? Some have interpreted these data as evidence for a deeply conserved petal identity program, suggesting that petal identity may have evolved once and just been redeployed to different positions in the flower (Bowman 1997). Others have held to the convergence model, that petals evolved many times but recruited similar genes to control their development in each case (Irish 2009). Discriminating among these alternatives may never be entirely possible but growing evidence seems to support a mixed model – the identity of true petals in the second whorl may be controlled by a commonly inherited genetic program in many cases but, at the same time, there are definitely genetic mechanisms that do not utilize the B gene program for promoting petaloid features in other positions of the flower (Rasmussen et al. 2009; and see also below).

Of course, understanding petal diversity only starts with questions related to organ identity. Across taxa, petals differ in every aspect of their morphology, including colour, symmetry and other aspects of shape, and presence or absence of nectaries. Our knowledge of the genetic control of these aspects is very uneven. Few direct targets of the B class genes have been identified (Mara & Irish 2008; Sablowski & Meyerowitz 1998), but several loci have been implicated in the sculpting of petals, particularly factors controlling the balance between cell

division and cell expansion (reviewed Irish 2008). Among these is *APETALA2* (*AP2*), which was originally considered an A class gene but has subsequently been found to primarily affect petal development rather than identity (Drews et al. 1991; Litt, 2007). Unlike the other genes of the ABC model, *AP2* is a member of the AP2/EREBP family of transcription factors (Okamuro et al. 1997; Weigel 1995). Together with a related family member, *AINTEGUMENTA* (*ANT*), *AP2* contributes to petal initiation and size (Keck et al. 2003; Krizek et al. 2000).

Another major influence on petal shape is whether or not the flower is bilaterally or radially symmetric. In the case of the former, different petal morphologies are produced in different positions in the same whorl of the flower. Studies of the genetic control of bilateral symmetry in *Antirrhinum* (snapdragon) indicate that fine control of cell division patterns is also critical to this type of differentiation (Gaudin et al. 2000). Studies of other bilaterally symmetric species, particularly legumes, have provided evidence that very similar genetic pathways have evolved by co-opting homologs of the same genes (reviewed by Preston & Hileman 2009; Rosin & Kramer 2009). At the same time, studies in legumes have demonstrated the potential to uncover novel factors that may be influencing the internal symmetry of individual petals (Wang et al. 2008).

Further elaborations of petal morphology take a variety of forms but one component that appears to be a critical aspect of diversification is the presence of nectaries (Hodges 1997a; Hodges & Arnold 1995). These structures, which produce sugar-rich liquid that serve as rewards for visiting pollinators, are not restricted to petals and can be present on any organ in the flower. Although nectaries have evolved many times independently (Bernardello 2007), a study of the core eudicots surprisingly found that orthologs of the same gene, the YABBY transcription

factor *CRABS CLAW* (*CRC*), are associated with nectaries from diverse taxa (Lee et al. 2005). However, this correlation between *CRC* and nectaries does not appear to extend outside the core eudicot clade. When nectaries occur on the perianth organs, they are often associated with the development of spurs that show a close correlation between their morphology and the feeding structures of the flower's pollinator. Currently, no candidate loci have been associated with the development of naturally occurring spurs but an intriguing mutant in *Antirrhinum* may provide some insight. Transposon insertions near two KNOX transcription factors result in ectopic gene expression that appears to promote the development of spur-like structures (Golz et al. 2002). It will be very interesting to see whether KNOX gene expression is actually responsible for the normal development of spurs.

Aside from nectaries, petals use other morphological features to attract pollinators, particularly the interplay of colour and iridescence. The latter is usually produced by the presence of conical or papillated epidermal cells. In *Antirrhinum*, a MYB transcription factor, *MIXTA* (*MIX*), is critical to the formation of these cells as well as proper pollinator attraction (Glover et al. 1998; Martin et al. 2002; Whitney et al. 2009). *Antirrhinum* has also been an important model for the genetic dissection of colour production, particularly in regards to the upstream regulators, which are also members of the MYB gene family (Noda et al. 1994; Schwinn et al. 2006). Natural variation among these paralogs has been shown to control spatial patterns of colour production and colour differences (Schwinn et al. 2006; Whibley et al. 2006). The enzymatic pathways responsible for colour production, which are genetically downstream of the MYBs, have been well characterized in a number of systems including *Antirrhinum*, *Ipomoea* and, recently, *Aquilegia* (Martin & Gerats 1993; Rausher 2008; Whittall et al. 2006; Hodges & Derieg 2009).

This leads to the question of *why* petals are so diverse. Over a century of research, including that of Darwin, has shown that pollinator interactions play a major role in shaping petal characteristics. As we review below, research in *Aquilegia* has shown that much of the intra and inter-specific variation in petals is intimately associated with variation in primary pollinators. These associations are especially true for variation in nectar spur length and colour (Fig. 2A-D). Because specialization to different pollinators causes reproductive isolation, petals represent important adaptive features in *Aquilegia* that may play direct roles in speciation processes. Thus, dissecting the genetic basis of these traits will provide an understanding how adaptation and speciation proceeds.

2. Morphology & evolutionary history of *Aquilegia*

Aquilegia is a member of the family Ranunculaceae, which falls within the eudicot order Ranunculales (APG 2003; Fig. 3). This order is sister to the rest of the core eudicots (Hoot et al. 1999). *Aquilegia*, therefore, adds an important third data point to deep evolutionary comparisons between the monocot grasses and core eudicot models such as *Arabidopsis*, *Petunia* and *Antirrhinum*. *Aquilegia* flowers are unique among these model genetic systems in that they possess five types of floral organs instead of the typical four: petaloid sepals in the first whorl, petals with nectar spurs in the second whorl, four to seven whorls of stamens, one whorl of staminodia, and an innermost whorl of carpels (Kramer 2009). The development of these organs has been studied in detail, confirming close affinities between the stamens and staminodia (Tucker & Hodges 2005). Some of the floral features of *Aquilegia* are found across the Ranunculaceae, particularly the petaloid sepals and petal nectaries, while the petal spurs and

staminodia are recently evolved (Hodges & Arnold 1994a; Ro & McPheron 1997; Tucker & Hodges 2005). The petal spurs, in particular, have been the subject of considerable research due to their critical role in pollinator interactions (Hodges 1997b; Hodges & Arnold 1994a; Hodges et al. 2004). The spurs initiate relatively late in floral development, after the stamens have differentiated into filaments and anthers (Tucker & Hodges 2005). Beginning as an outpocketing close to the base of the concave petal, the spur does not elongate substantially until the last phases of floral development, reaching a final length of anywhere from 1-2mm to 10-12 cm (Hodges and Arnold 1995; Tucker & Hodges 2005). The nectary develops inside the distal tip of the spur. It appears that diversity in floral spurs, along with other aspects of floral morphology such as orientation and colour, have facilitated the rapid radiation of *Aquilegia* species in less than 2 million years (Hodges 1997b; Kay et al 2006; Whittall & Hodges 2007). This recent evolutionary history has made the genus an excellent model for understanding speciation via pollinator diversification (Hodges et al. 2004). Other interesting morphological and physiological features of *Aquilegia* include compound leaves, a perennial life cycle, vernalization-based control of flowering time, and adaptations to a variety of environments including alpine and desert.

3. Genetic & Genomic Resources for *Aquilegia*

To facilitate the genetic dissection of traits in *Aquilegia*, genomic resources have been, and are being, developed for the genus. One consequence of the recent and rapid divergence of *Aquilegia* species is extreme similarity at the DNA level among species (Hodges & Arnold 1994a; Kay et al. 2006; Whittall et al. 2006; Whittall & Hodges 2007). Thus, resources developed from one species are often readily transferable to most other species of the genus (Whittall et al. 2006;

Yang et al. 2005). This transferability across species that vary markedly in ecology and morphology is a great advantage to *Aquilegia* as a model system.

At the core of the development of new model species is the creation of an EST database (Abzhanov et al. 2008). An EST database is ideal for the identification of candidate genes (e.g., Hodges & Derieg 2009), the development of expression arrays and can be used for phylogenomics (Abzhanov et al. 2008). Such a database becomes increasingly useful as the full transcriptome becomes represented. For the *Aquilegia* EST database (the Aquilegia Gene Index, AqGI, http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=aquilegia), mRNA was isolated from a broad range of tissues and developmental stages including vegetative and floral apical meristems, flowers from early buds through anthesis, as well as leaves and roots from hybrids of *A. formosa* X *A. pubescens*. This diversity and emphasis on flowers was designed to capture a large portion of the transcriptome and those genes involved with floral development in particular. In addition, the mRNA was selected for full-length transcripts and normalized in order to obtain as many transcripts with their entire, or nearly entire, sequence.

The current version of the AqGI (release 2.1) contains over 85,000 sequences that assemble into 13,556 tentative consensus (TC) sequences and 7,278 singletons. This is likely to represent a high percentage of *Aquilegia* genes, given that *Arabidopsis* currently has just over 27,000 protein-coding genes (TAIR9). The *Aquilegia* TCs average 1,293 bp in length (Fig. 4), close to the mean length found for eukaryotic genes (1,346 bp; Xu et al. 2006). This similarity suggests that most of the TCs in the AqGI represent near full-length transcripts. Many of the genes have been classified using GO vocabularies to various molecular functions, biological processes and

cellular components (Fig. 5A-C). As expected, general categories such as 'catalytic activity' and 'cellular processes' are highly represented, but there is also a broad range of classifications highlighting the diversity of genes represented. Thus, the AqGI provides an excellent resource for searching for homologs of specific genes and also for transcriptome studies using either oligonucleotide arrays or next generation sequencing.

In addition to the AqGI, other genetic and genomic tools are being developed for *Aquilegia*. Currently there are three BAC libraries, two for *A. formosa* and one for *Aquilegia coerulea* Goldsmith, an inbred horticultural line. The *A. formosa* BAC libraries have been fingerprinted and assembled into a physical map (https://www.genome.clemson.edu/activities/projects/aquilegia/pmap/). These maps will greatly aid in the assembly of a complete genome sequence, which is currently in production at the DOE Joint Genome Institute. A complete genome sequence will be especially useful for identifying the genes underlying genetic variants.

While the above resources are ideal for identifying candidate genes, in order to test if they actually influence specific traits, a functional assay is essential (Abzhanov et al. 2008). Luckily, we have been able to adapt a reverse genetic tool, virus-induced gene silencing (VIGS), for use in *Aquilegia* (see Gould & Kramer 2007 for detailed protocol). This approach uses the tobacco rattle virus to induce RNAi-based silencing of targeted genes (Burch-Smith et al. 2004). This method is especially useful since the effect of repressing gene expression can be observed in a matter of weeks, but it also has a number of disadvantages (Gould & Kramer 2007). These include the fact that genes can only be down-regulated, not over-expressed, and the fact that

silencing often occurs in clonal sectors of varying size rather than constitutively in all tissues. In order to address these problems, we are working to develop stable transformation as a complement to VIGS. We believe that *Aquilegia* may be a good candidate for dip transformation due to the fact that its carpels do not close until quite late in development (Tucker and Hodges 2005), but we are also pursuing the possibility of tissue culture transformation.

4. Developmental genetics

The diverse floral organ types of *Aquilegia* bring several evolutionary questions to mind. Are the B class genes responsible for petaloid aspects in the first whorl sepals? If they are, how is the identity of the second whorl petals distinguished from that of the petaloid sepals? What genetic pathways are responsible for the development of nectar spurs on the petals? How is the identity of the fifth organ type, the staminodia, determined? Answering all of these questions starts with identifying homologs of the floral organ identity genes from *Aquilegia* as well as related taxa.

Multiple studies of the MADS box genes have found that they are highly prone to retain duplicate copies, which may lead to divergences in gene function (Kramer & Zimmer 2006; Litt & Irish 2003; Zahn et al. 2005a). For this reason, it is critical to first establish whether paralogs are present and, if so, when the duplication events occurred. In the case of *Aquilegia*, we have detected three *AP3* homologs and one *PI* (Kramer et al. 2003). The multiple *AP3* paralogs are derived from relatively ancient events that predated the diversification of the Ranunculaceae and, most likely, the bulk of the order Ranunculales (Kramer et al. 2003; Rasmussen et al. 2009). In keeping with these ancient paralogs, the expression patterns of the *AP3* loci, termed *AqAP3-1*, *AqAP3-2* and *AqAP3-3*, are quite different from one another (Fig. 1) (Kramer et al. 2003; Kramer

et al. 2007; Rasmussen et al. 2009). *AqAP3-1* is broadly expressed in petal, stamen and staminodium primordia at very early stages but, as soon as the carpels initiate, this domain begins to contract until it only comprises the staminodia (Kramer et al. 2007). The second paralog, *AqAP3-2*, is turned on slightly later in a region covering the stamens and staminodia but not the petals. At slightly later stages, it shifts to exclude the staminodia but comes on in the petals. Lastly, *AqAP3-3* is very specifically expressed in the petals throughout their development. The other B gene homolog, *AqPI*, is broadly expressed throughout the petals, stamens and staminodia. This is in keeping with the finding that the AqPI protein heterodimerizes specifically with all three AP3 proteins (Kramer et al. 2007). The spatially and temporally distinct expression patterns of each *AP3* paralog are suggestive of subfunctionalization (Force et al. 1999).

Subfunctionalization is an evolutionary process whereby complex, ancestral functional repertoires become genetically parsed among duplicate gene copies. In the case of these B class gene homologs, it would appear that an ancestral expression domain encompassing the petals and stamens throughout development has become subdivided such that one copy is primarily expressed at early stages (*AqAP3-1*), another is primarily in stamens (*AqAP3-2*) and the third is limited to petals (*AqAP3-3*) (Kramer et al. 2007). This situation is made more complex by the presence of the staminodia, which are a novel organ type that evolved very recently (Ro & McPheron 1997; Tucker & Hodges 2005). In these organs, we see specific late expression of the *AqAP3-1* paralog to the exclusion of the other two (Kramer et al. 2007). This suggests an attractive hypothesis whereby the evolution of this new organ identity has been facilitated by the presence of B class gene paralogs– an example of neofunctionalization following gene duplication (Force et al. 1999).

Another important point is in regard to the petaloid sepals. The expression of the B gene homologs can be detected in these organs at later stages using RT-PCR but *in situ* hybridization at early developmental stages does not detect *AP3* and *PI* expression (Kramer et al. 2003; Kramer et al. 2007). Given that studies in core eudicot models indicate that expression is required from inception to determine proper organ identity (Bowman et al. 1989; Zachgo et al. 1995), these results indicate that the loci are not contributing to the identity of the petaloid sepals. The data do leave open the possibility that later stage expression could be important for promoting petaloid features, which actually appear fairly late in development (Kramer et al. 2007).

These hypotheses require rigorous functional testing in order to determine whether the loci are actually required for the identity of petals, stamens and staminodia as well as the late petaloidy of the sepals. Application of VIGS to the *AqPI* locus resulted in dramatic homeotic transformations of petals into sepals and both stamens and staminodia into carpels (Kramer et al. 2007). A strongly modified phenotype would be expected given the fact that AqPI is required for the function of all three AqAP3 paralogs due to their heterodimerization. These findings demonstrate that the B class genes are, in fact, essential to petal, stamen and staminodium identity. Interestingly, however, there was no effect on sepal identity and the conical epidermal cells, which are among the hallmarks of petaloidy, were not affected. This leads us to believe that the B gene homologs are not critical to either the identity of the sepals or their petaloidy. This finding is in keeping with studies of other taxa with petaloid sepals that have similarly

found that B gene homologs are not expressed in these organs (Geuten et al. 2006; Jaramillo & Kramer 2004; Park et al. 2004; Park et al. 2003).

Several research directions immediately suggest themselves from these results. First, we would like to use VIGS to knockdown each of the three *AP3* paralogs in turn. This will determine their degree of redundancy and whether they have specific functions in different whorls. One challenge will be the specific targeting of each paralog separate from the others, which may not be possible using VIGS, although other studies indicate a high degree of specificity (Liu et al. 2004). Another matter of considerable interest is how petaloidy is actually promoted in the sepals, since this does not appear to involve B gene homologs. To pursue this question, we are taking a mixed approach of both forward and reverse genetics/genomics. In the context of reverse genetics, which relies on candidate genes, we will examine two loci called *AqSEP1* and *AqSEP2*. These genes are homologs of the *Arabidopsis* E class genes *SEPALLATA1/2/4* and show sepal-specific expression in *Aquilegia* (Kramer et al. 2007). When *AqPI* is silenced and the petals are transformed into sepals, the expression of these genes expands into the second whorl, suggesting that they are closely associated with sepal identity. We will also take a gene discovery approach that utilizes next generation sequencing and available horticultural homeotic mutants of *Aquilegia* (Fig 2F). By comparing the floral transcriptome of flowers composed almost exclusively of sepals to that of flowers composed primarily of petals, we hope to be able to identify sepal-specific gene networks that are involved in promoting petaloidy. This experiment has the added benefit of identifying petal-specific pathways, which could include genes involved in nectary and spur development. As noted above, the *Aquilegia CRC* ortholog does not appear

to be involved in nectary development (Lee et al. 2005) so we do not currently have candidate genes for the nectary.

For the spur itself, we are considering an array of candidate loci including KNOX genes and several genes controlling the balance between cell division and expansion such as *JAGGED*, *BIG PETAL*, and *AINTEGUMENTA* (reviewed Irish 2008). This line of research can be explored on an evolutionary scale as well. Since the nectar spur is specific to *Aquilegia*, candidate loci for its development can be studied comparatively relative to outgroup taxa in order to understand how these genetic modules were recruited to function in spur development. Within the genus, the critical question is how diversification of spur length and morphology is controlled at the genetic level. Candidate genes for spur development will, therefore, also become candidates for QTL studies of natural variation in spur morphology. Previous genetic studies using interspecies hybrids have suggested that there may be relatively few loci controlling spur initiation with higher numbers of genes modulating length (Prazmo 1965).

5. Phylogeny, Ecology & Pollination biology

While *Aquilegia* is ideal for dissecting the genetic basis of floral organ differentiation, the genus has also long been noted for the diversity of shape and colour of its petals in association with variation in pollinators. For example, just among North American species, spur length varies over a 16-fold range (Whittall & Hodges 2007). In addition, spurs can be straight or highly curled and the diameter of the tube varies, especially at the opening, from fairly broad to quite narrow (Fig. 6). Petal blades vary similarly in length (both absolute and relative to spur length), width, and orientation to the floral axis (Fig. 2A-D, 6). Finally, the colour of the spurs varies

tremendously including red, blue, purple, violet, yellow and white. Both the spur and the blade may be the same colour (Fig. 6A-C) or, often, the blade may be wholly or partially contrasting with the spur (Fig. 6D-F). Much of this variation in petals has been correlated with the types of pollinators visiting *Aquilegia,* thus, establishing these traits as adaptations. The genetic dissection of this variation is therefore of particular interest as debate continues over the types of mutations and the genes involved in adaptation (e.g., Hoekstra & Coyne 2007).

Darwin himself was interested in the association of petal spurs with their pollinators and proposed a co-evolutionary model to account for the exceptionally long spurs of *Angraecum sesquipedale* (Darwin 1862). He envisioned reciprocating evolution between a pollinator's tongue and the plant's spur; when the average tongue length is shorter than spur length, longer-tongued individuals have an fitness advantage by gaining more nectar rewards, and when the average spur length is shorter than tongue length, longer spurred individuals have a fitness advantage by having more pollen dispersed and received. This reciprocating selection pressure would then lead to a gradual lengthening of both tongues and spurs. Alternatively however, evolution may be one-sided with floral-spurs evolving to fit the tongue-lengths of pollinators, which do not evolve in response to spur-length (Wasserthal 1997). This hypothesis suggests that increases in spur length will occur during transitions between primary pollinators with different tongue lengths (Wasserthal 1997). Recently these hypotheses were tested phylogenetically using the North American *Aquilegia* species (Whittall & Hodges 2007). Transitions between pollination classes were found to be directional (bee to hummingbird and hummingbird to hawkmoth) with concomitant increases in spur length. In addition, models of the pace of spur-length evolution strongly favored those where evolution occurred primarily at the time of

speciation. Thus, in *Aquilegia*, spur-length evolution has likely proceeded primarily due to rapid adaptation to the already established tongue-lengths of its primary pollinators rather than in a co-evolutionary race as envisioned by Darwin and others (Whittall & Hodges 2007; Hodges & Whittall 2008).

The match between spur and pollinator tongue length has been studied in depth in *Aquilegia formosa* and *A. pubescens* along with a number of other petal characteristics. Over 50 years ago, Verne Grant used these two species, as the first example of how floral characters could be associated with specific pollinator visitation and thus affect the degree of reproductive isolation between species (Grant 1952). In particular, Grant noted the differences between the species in the colour and length of the petal spurs and blades. He found that hummingbirds visited *A. formosa* with short, red and yellow petals while hawkmoths visited *A. pubescens* with long, white or pale-yellow petals. Given that these two species are highly intercompatible, Grant concluded that these petal traits were a major source of reproductive isolation between these species due to their affect on pollinator discrimination (Grant 1952, 1976). Chase & Raven (1975) challenged Grant's conclusion because they observed instances of visitation by hummingbirds and hawkmoths to both species. Grant (1976) then disputed Chase & Raven's findings noting that their observations were not made when both species were blooming simultaneously, the situation most relevant to reproductive isolation. Subsequently, simultaneous observations of both species in natural stands and in experimental arrays showed that hummingbirds and hawkmoths do strongly differentiate between these species (Fulton & Hodges 1999; Hodges et al. 2002). Furthermore, manipulative experiments showed that spur length strongly affects pollen removal (and likely deposition on stigmas as well), while flower colour

and orientation strongly affect pollinator visitation (Fulton & Hodges; Hodges et al 2002) and these traits show steeper clines than neutral genetic markers (Hodges & Arnold 1994b). Thus both petal colour and spur-length can directly affect reproductive isolation and speciation in *Aquilegia*.

In addition to spur length, the colour of the petals has been strongly linked to different pollination syndromes in *Aquilegia* (Hodges et al. 2004; Whittall & Hodges 2007). Species with predominantly bee pollination tend to be blue-purple, while those with strong hummingbird visitation are red and those with hawkmoth visitation tend to be white or yellow (Fulton & Hodges 1999; Hodges et al. 2004; Brunet 2009). The adaptive nature of these colour differences has been best studied with hawkmoths, which discriminate against red flowers in favor of white ones (Hodges et al. 2004). In *A. coerulea*, where flower colour can be polymorphic within populations (blue and white), populations with consistent hawkmoth visitation tend to have whiter flowers (Brunet 2009) and in populations with variable abundance of hawkmoths, white flowers set more seed when they are present (Miller 1981). Collectively these studies strongly support the evolution of light-coloured flowers as an adaptation to hawkmoth pollination.

Anthocyanin pigments produce the blue/purple and red pigments of Aquilegia flowers while yellow is produced by carotenoids (Taylor 1984). Thus a major transition in *Aquilegia* has been from flowers producing anthocyanins (hummingbird pollination) to those lacking these pigments (hawkmoth pollination) (Whittall et al. 2006). Given that petal colour has been a major focus of genetic studies since Mendel's original experiments with the garden pea, a great deal is known about the genes underlying the anthocyanin biochemical pathway (ABP) including the

transcriptional regulators (reviewed in Hodges and Derieg 2009). The core pathway consists of only six enzymatic steps and these loci, along with their transcriptional regulators, have been the subject of evolutionary studies in a number of species (Martin & Gerats 1993; Rausher 2008; Streisfeld & Rausher 2009). However, in addition to these genes, a number of other enzymatic pathways (e.g., those producing flavones and flavonols) intersect with the core pathway and can cause flux away from anthocyanin production and thus may influence flower colour (see Hodges & Derieg 2009).

Phylogenetic analysis indicates multiple independent transitions to hawkmoth pollination in *Aquilegia*, all of which involve the loss of anthocyanins from petals. This distinctive pattern has allowed us to test whether convergence in phenotype is precipitated by convergence at the molecular level (Whittall et al. 2006). Across multiple independent losses of anthocyanins, Whittall et al. (2006) found that most involve down-regulation of multiple genes late in the core ABP. This finding, along with multiple studies suggesting that the losses of floral anthocyanins in *Aquilegia* is due to single QTL (Prazmo 1965; Taylor 1984; Hodges et al. 2002), point to mutations in translational regulators as common causes of these evolutionary transitions. However, ideally, all the genes in the ABP, the pathways that intersect with the ABP, and their translational regulators should be evaluated. Using the AqGI and rtPCR, Derieg & Hodges (2009) identified 34 candidate genes for the entire flavonoid pathway and its regulators. To monitor the expression of all of these genes with traditional methods such as rtPCR would be a daunting task. However, with the advent of next-generation sequencing, it should be possible to monitor both the expression and sequence variation of all of these genes simultaneously by sequencing directly from mRNA. Another future direction will be to establish that the candidate

genes, especially the translational regulators, function as predicted. This could be accomplished utilizing VIGS to target these genes and determining if anthocyanin production is suppressed (Gould & Kramer 2007).

While the shape and colour of the petals of *Aquilegia* clearly have been central to adaptation to pollinators and speciation in the genus, the evolution of the nectar spur itself is especially intriguing. The origin of nectar spurs across numerous groups of flowering plants has been correlated with species diversification and termed a 'key innovation' (Hodges & Arnold 1995; Hodges 1997; Kay et al. 2006). Thus, as noted above, an important goal is to identify the genetic program that generates the spur itself. Prazmo (1961) performed now classic experiments by crossing the spurless columbine, *A. ecalcarata*, and spurred species. When she classified F2 offspring from these crosses as spurred/non-spurred, Prazmo found simple Mendelian ratios suggesting that only one or two QTL were responsible for the presence of a spur. Plants that did possess petal spurs had a broad range of spur length suggesting a more complex and polygenic control of this aspect of spur development. If *A. ecalcarata* represents the ancestral character state of lacking spurs, then these experiments would indicate that spurs could evolve through one or two mutations (Gottleib 1984; Orr & Coyne 1992). However, phylogenetic analysis suggests that *A. ecalcarata* is nested within *Aquilegia* and that it represents the loss of petal spurs rather than the ancestral state (Hodges and Arnold 1995). Thus the origin of nectar spurs may have required a more complex set of genetic changes. Regardless, comparisons of gene expression patterns between *A. ecalcarata* and spurred species, as well as between progeny of segregating populations will likely provide strong candidates for important genes involved with the development of this novel trait.

6. Deeper questions about the assessment of homology in petals

As discussed in the Introduction, petals are thought to have evolved many times independently across the angiosperms. These ideas about independent derivations, particularly in regards to andropetals, have been greatly elaborated in the family Ranunculaceae. Starting with the earliest morphological studies, the second whorl organs of many members of the family were set apart as "nectar leaves" or, more poetically, "honey leaves" (Prantl 1887). Although these organs are sterile and positioned in the second whorl of the flower, thereby fitting the broader definition of petals, they were considered to have evolved completely independently on many occasions, each case resulting from a sterilization of outer stamens (Hiepko 1965; Prantl 1887; Tamura 1965; Worsdell 1903). Evidence for these derivations is drawn from vascular patterns, developmental and morphological similarities, and homeotic interconversions. Such explicit hypotheses make the Ranunculaceae a very attractive group for investigating the genetic evidence for independent petal derivations. One caveat, however, is the problem with interpreting expression of B gene homologs – does "conserved" expression support a deeply conserved petal identity program or does it simply indicate independent recruitment of homologous genes? In the case of *Aquilegia*, there are two important factors. First, we have moved beyond mere expression data to demonstrate that the B gene homologs are essential to petal identity (Kramer et al. 2007). Second, the *AP3* duplications provide us with a unique and fortuitous marker for petal identity: the *AqAP3-3* ortholog is petal specific in its expression. This condition could have arisen in several different ways, however. If the petals of *Aquilegia* evolved independently relative to those of other Ranunculaceae, then *AqAP3-3* could have been recruited to its petal-specific domain quite recently. Under this model, we would not expect orthologs of *AqAP3-3* to have

similar expression patterns in other genera. On the other hand, if a commonly inherited genetic program controls petals in the Ranunculaceae, then other genera could show the same petal-specific expression.

We used RT-PCR to survey gene expression patterns among orthologs of *AqAP3-1*, *-2* and *-3* across thirteen members of three families of the Ranunculales, which was combined with previous studies of seven genera from additional ranunculid families. We found that among taxa bearing petals, seven of eight genera of the Ranunculaceae and both of the surveyed Berberidaceae genera have *AqAP3-3* orthologs that are specifically expressed in the petals (Rasmussen et al. 2009). Furthermore, members of this gene lineage tend not to be expressed in flowers that lack petals, such as those of *Caltha* and *Thalictrum*. We believe that the most parsimonious explanation for this pattern is that a commonly inherited petal identity program is functioning across the Ranunculaceae and Berberidaceae families. Under this model, the apetalous taxa of the two families likely represent petal loss events rather than an ancestral condition. One important caveat, however, is that there is at least one case where petals have clearly reappeared from an apetalous ancestor, that being the *Atragene* and *Naraveliopsis* sections of the genus *Clematis* (Miikeda et al. 2006).

This hypothesis regarding process homology among Ranunculaceae petals contradicts over a century of botanical theory and, therefore, must be thoroughly evaluated. Functional tests on *Aquilegia AqAP3-3* as well as its orthologs in several other genera would be ideal. At the same time, it is equally important to reconsider certain typologies regarding the "nectar leaves" of the Ranunculaceae. For example, the common presence of nectaries on Ranunculaceae petals has

been considered a feature that associates the petals with the stamens (Prantl 1887; Worsdell 1903). However, nectaries are never present on the stamens of flowers that lack petals (Tamura 1993), which would seem to suggest that their common presence on petals is a trait that associates the petals with each other rather than the petals with stamens. Given our already detailed understanding of plant developmental genetics, it is a timely endeavor to reevaluate such long-held concepts, which were largely developed to provide structure to a field that had few consistent characters to use for assessments of the homology and evolutionary derivation of floral organs.

7. Concluding Remarks

Aquilegia is not simply a model species but a model genus, with up to 70 different species that can be used to address a wide range of biological questions. We have focused on the evolution and ecology of petals and petaloid organs since they offer an opportunity to explore the full spectrum of genetics to morphology to evolutionary diversification to ecology of biotic interactions. However, future research into the evolutionary genetics of nectar spurs and floral colour promises even more exciting results. Of course, there is more to *Aquilegia* than just petals. In addition to the novel staminodia, their compound leaves, cymose inflorescences, vernalization response, soil adaptations and perenniality are all worthy of investigation. The development of genomic data for a wide range of *Aquilegia* species will allow these characteristics to be fully explored by a growing community of researchers.

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Fig. 1. A. The classic ABC model with the addition of the E function. B. The corresponding ABCE genes from *Arabidopsis*. The A class genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) specify sepals (SEP) and with the B class genes, *APETALA3* (*AP3*) and *PISTALLATA* (*PI*), specify petals (PET). The B class genes with the C class gene *AGAMOUS* (*AG*) specify stamens (STA) and the C class gene alone specifies carpels (CAR).C. and D. The modified ABC model of Aquilegia based on expression studies of the B gene homologs. C. Corresponds to early developmental stages while D. reflects expression after carpel initiation.

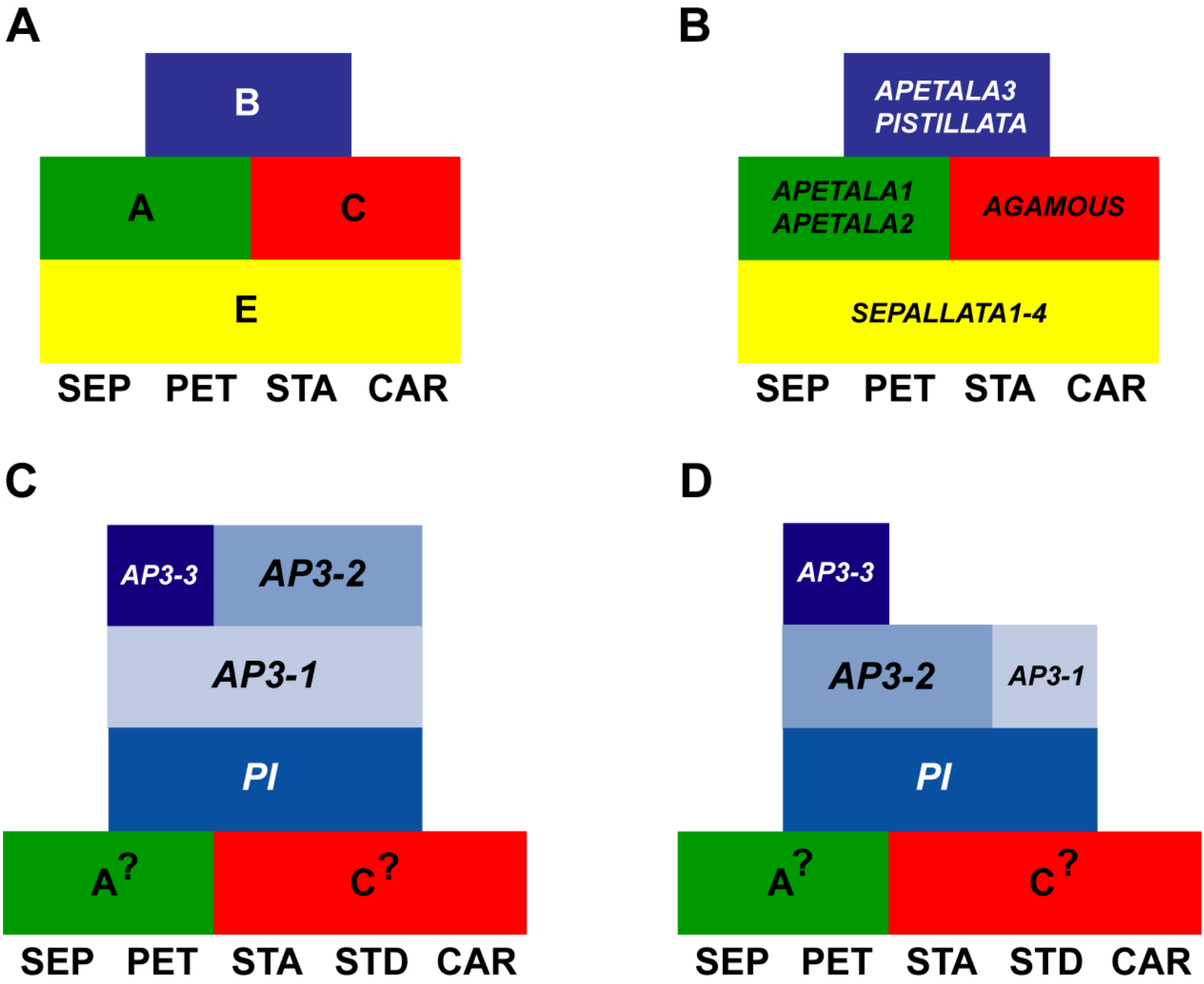
Fig. 2. Floral variation across natural species and one cultivar of *Aquilegia*. A. *A coerulea*. B. *A. shockleyi*. C. *A. pubescens*. D. *A. chrysantha*. E. *A. coerulea* var. daileyae, which lacks spurs. F. *A. vulgaris* 'Black Tower', which has stamens transformed into petals. Photos: A, Nathan Derieg; B-E, Scott Hodges; F, Elena Kramer.

Fig. 3. Simplified phylogeny of the angiosperms based on Moore et al. (2007) showing the position of *Aquilegia* relative to other major model systems.

Fig. 4. Frequency distribution of the length of tentative consensus (TC) sequences in the AqGI.

Fig. 5. Classification of sequences from the AqGI to GO vocabularies. A. Molecular Function, B. Biological Processes, C. Cellular Component.

Fig. 6. Comparison of individual petals among species of *Aquilegia*. a. *A. longissima*, b. *A. pinetorum*, c. *A. chrysantha*, d & e, *A. formosa*, f. *A. flabellata*.


A
B
A
C
E
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B
APETALA3
PISTILLATA
APETALA1
APETALA2
AGAMOUS
SEPALLATA1-4
SEP PET STA CAR
C
AP3-3
AP3-2
AP3-1
PI
A?
C?
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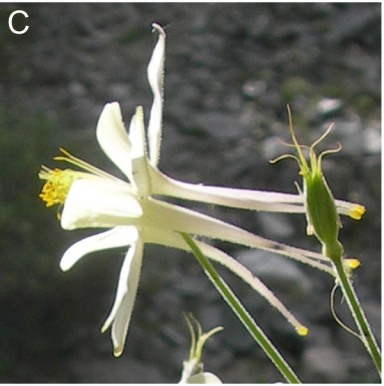
PI
A?
C?
SEP PET STA STD CAR


A

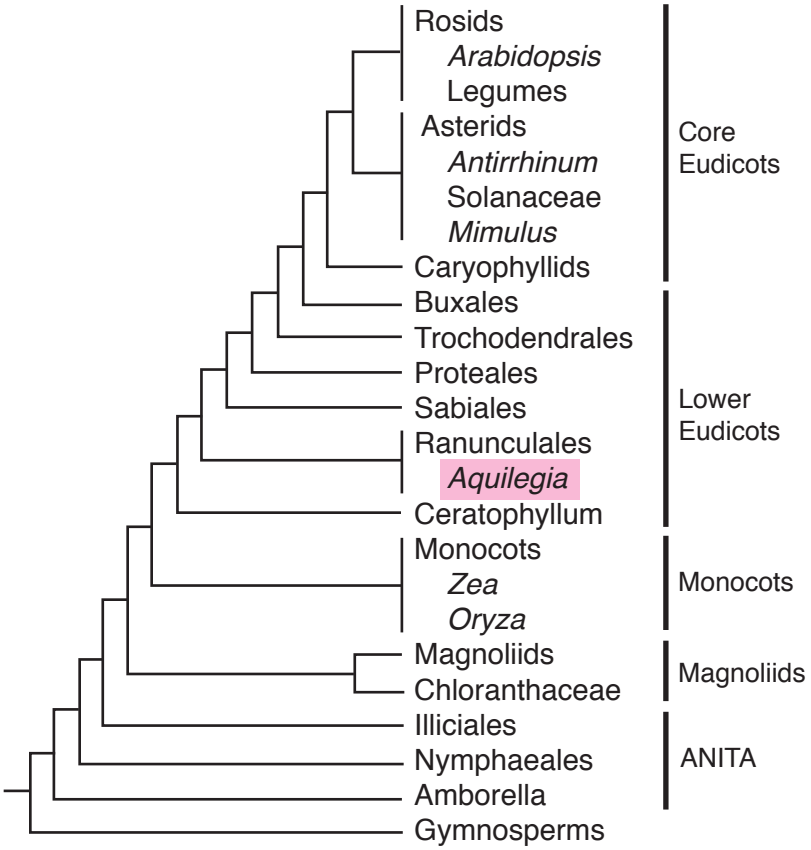

B


C


D


E


F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Aquilegia
Ceratophyllum
Monocots
Zea
Oryza
Magnoliids
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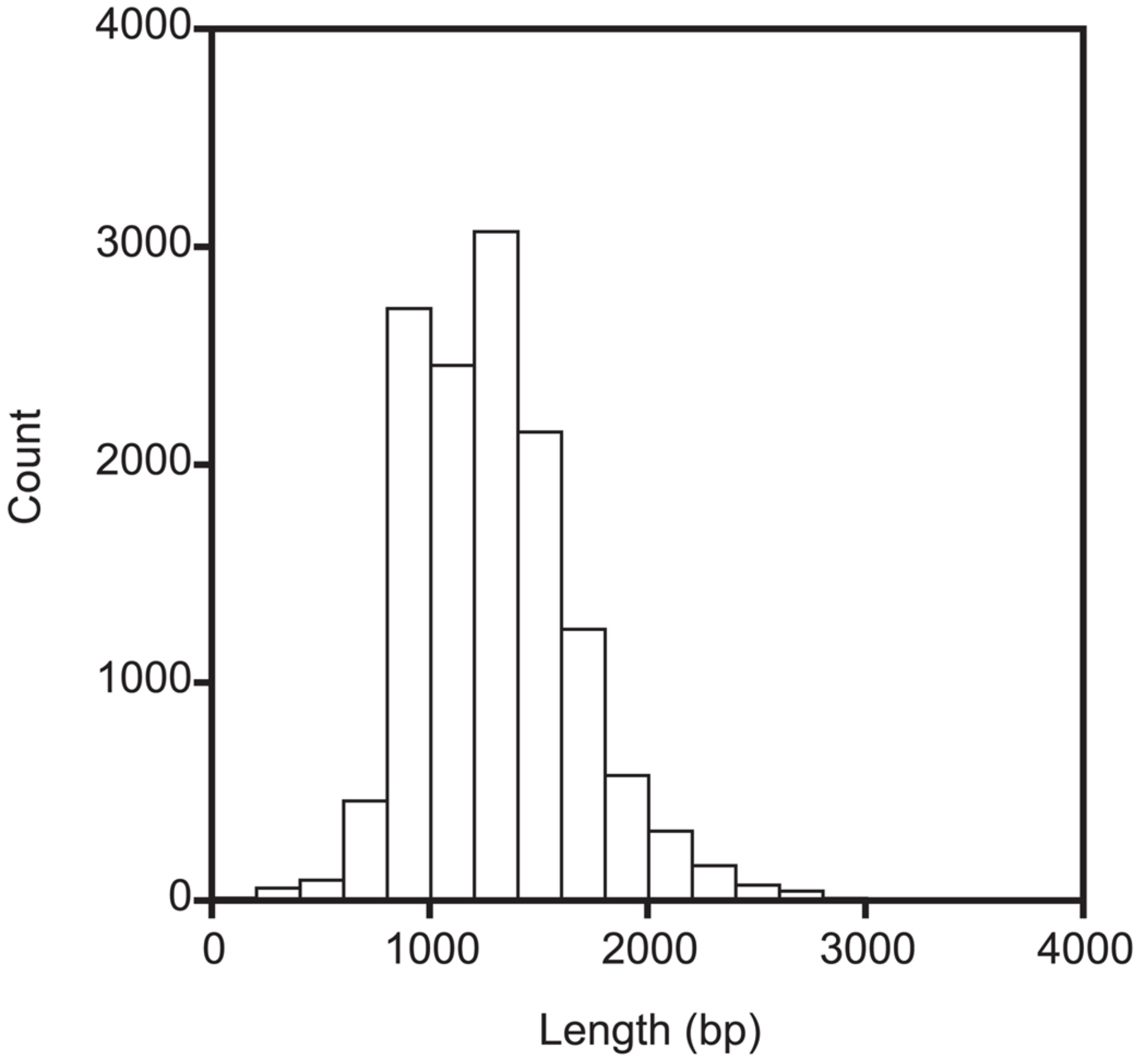
ANITA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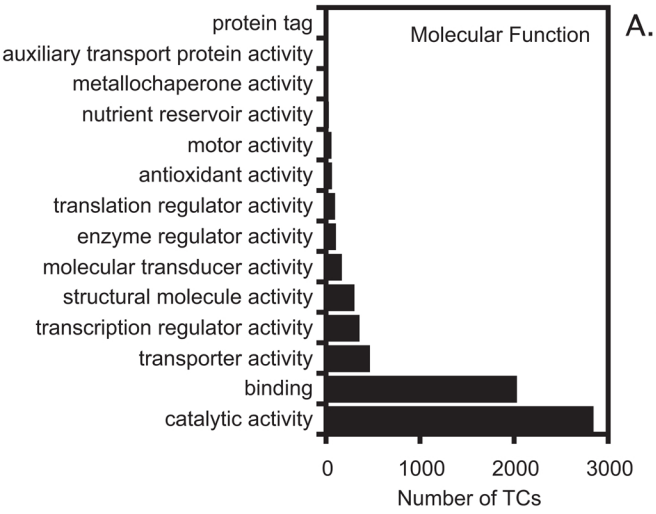
Molecular Function
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metallochaperone activity
nutrient reservoir activity
motor activity
antioxidant activity
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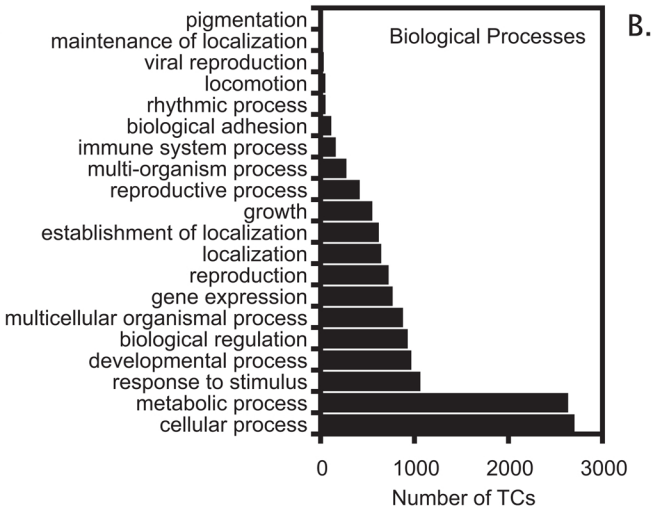
Number of TCs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multi-organism process
reproductive process
growth
establishment of localization
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developmental process
response to stimulus
metabolic process
cellular process
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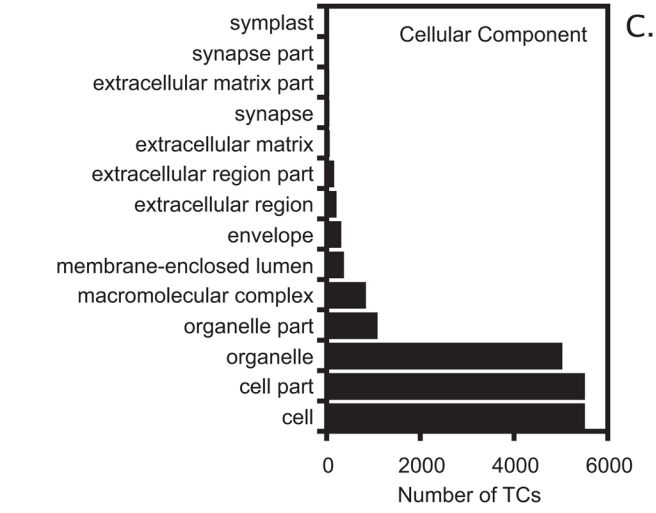
Cellular Component
C.
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extracellular matrix part
synapse
extracellular matrix
extracellular region part
extracellular region
envelope
membrane-enclosed lumen
macromolecular complex
organelle part
organelle
cell part
cell
0
2000
4000
6000
Number of TCs


A
B
C
F
D
E
1 cm